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**Citation for published version:**

Huang, Y-W, Patterson, AR, Opriessnig, T, Dryman, BA, Gallei, A, Harrall, KK, Vaughn, EM, Roof, MB & Meng, X-J 2012, 'Rescue of a porcine anellovirus (torque teno sus virus 2) from cloned genomic DNA in pigs', *Journal of Virology*, vol. 86, no. 11, pp. 6042-54. <https://doi.org/10.1128/JVI.00175-12>

**Digital Object Identifier (DOI):**

[10.1128/JVI.00175-12](https://doi.org/10.1128/JVI.00175-12)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Publisher's PDF, also known as Version of record

**Published In:**

Journal of Virology

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# Rescue of a Porcine Anellovirus (Torque Teno Sus Virus 2) from Cloned Genomic DNA in Pigs

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**Anelloviruses are a group of single-stranded circular DNA viruses infecting humans and other animal species. Animal models combined with reverse genetic systems of anellovirus have not been developed. We report here the construction and initial characterization of full-length DNA clones of a porcine anellovirus, torque teno sus virus 2 (TTSuV2), *in vitro* and *in vivo*. We first demonstrated that five cell lines, including PK-15 cells, are free of TTSuV1 or TTSuV2 contamination, as determined by a real-time PCR and an immunofluorescence assay (IFA) using anti-TTSuV antibodies. Recombinant plasmids harboring monomeric or tandem-dimerized genomic DNA of TTSuV2 from the United States and Germany were constructed. Circular TTSuV2 genomic DNA with or without introduced genetic markers and tandem-dimerized TTSuV2 plasmids were transfected into PK-15 cells, respectively. Splicing of viral mRNAs was identified in transfected cells. Expression of TTSuV2-specific open reading frame 1 (ORF1) in cell nuclei, especially in nucleoli, was detected by IFA. However, evidence of productive TTSuV2 infection was not observed in 12 different cell lines transfected with the TTSuV2 DNA clones. Transfection with circular DNA from a TTSuV2 deletion mutant did not produce ORF1 protein, suggesting that the observed ORF1 expression is driven by TTSuV2 DNA replication in cells. Pigs inoculated with either the tandem-dimerized clones or circular genomic DNA of U.S. TTSuV2 developed viremia, and the introduced genetic markers were retained in viral DNA recovered from the sera of infected pigs. The availability of an infectious DNA clone of TTSuV2 will facilitate future study of porcine anellovirus pathogenesis and biology.**

Anelloviruses are small, single-stranded, circular DNA viruses that infect a wide range of animal species, from humans to domestic animals, including pigs (13, 34). Most recently, all human and other animal anelloviruses have been assigned to a newly established family, *Anelloviridae*, that includes nine genera (3). Human anelloviruses include torque teno virus (TTV), torque teno minivirus (TTMV), and torque teno midivirus (TTMDV), which belong to three different genera. Human TTV, TTMV, and TTMDV are nonenveloped spherical viruses with DNA genomes 3.6 to 3.9, 2.8 to 2.9, and 3.2 kb long, respectively (33). These three groups of human anelloviruses show a high degree of genetic diversity, and a high prevalence of infections with TTV, TTMV, and TTMDV in human populations has been documented worldwide (32, 33). On the other hand, porcine anellovirus or torque teno sus virus (TTSuV) is assigned to a new genus, *Iotatorquevirus*, comprising two species (TTSuV1 and TTSuV2), each also characterized by high genetic diversity with a genomic size of approximately 2.8 kb (16, 31). TTSuV1 and TTSuV2 are highly prevalent in pig populations in many countries (9, 20, 28).

Human and porcine anelloviruses have the same genomic structure, which consists of at least four presumed open reading frames (ORFs), ORF1, ORF2, ORF1/1, and ORF2/2, as well as a short stretch with high GC content in the untranslated region (UTR) (5, 16, 36, 38). The transcription pattern and related translational products of human TTV genogroup 1 have been experimentally determined by using two full-length TTV DNA clones (5, 30, 38). It was shown that the human TTV genome expresses three or more spliced mRNAs encoding at least six proteins, ORF1, ORF2, ORF1/1, ORF2/2, ORF1/2, and ORF2/3 (5, 30). The transcriptional analysis and protein expression profile using cloned full-length genomic DNA have not been reported for TTSuV. The ORF1 of TTSuV is believed to encode

a viral capsid and replication-associated protein (5, 16, 36). IgG antibodies against ORF1 of TTV and TTSuV have been detected in human and pig sera, respectively (15, 17, 37).

The pathogenic potential of anellovirus is still controversial. Currently, human TTV is not considered to be directly associated with a particular disease, although recent studies suggested that TTV may serve as an immunological trigger of multiple sclerosis (26). Similarly, whether TTSuV is associated with a swine disease is still debatable. TTSuV1 was shown to partially contribute to the experimental induction of porcine dermatitis and nephropathy syndrome and postweaning multisystemic wasting syndrome (PMWS or porcine circovirus [PCV]-associated disease, PCVAD) in a gnotobiotic pig model (6, 21). PMWS-affected pigs in Spain had a higher prevalence and greater loads of TTSuV2 than did non-PMWS-affected pigs (1, 20). Moreover, a significantly lower level of anti-TTSuV2 antibody was found in PCVAD-affected pigs than in non-PCVAD-affected pigs (15). However, results from other studies did not support a direct association of TTSuV1 or TTSuV2 with PCVAD or an association of type 2 PCV (PCV2) and TTSuV with porcine reproductive failures (10, 23, 39; Y. W. Huang et al., submitted for publication).

Due to the lack of a cell culture system to propagate anellovi-

Received 21 January 2012 Accepted 22 March 2012

Published ahead of print 4 April 2012

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Supplemental material for this article may be found at <http://jvi.asm.org/>.

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doi:10.1128/JVI.00175-12

rus, little is known regarding the molecular biology and pathogenesis of anelloviruses. In order to definitively characterize diseases associated with anellovirus infection, an appropriate animal model is needed. Since infections with multiple different genotypes or subtypes of human TTV or TTSuV are common events (9, 16, 32), a biologically pure and isolated form of a specific anellovirus generated from a full-length infectious DNA clone is also required for a study of the pathology due to a single phenotype. Although infectious DNA clones of human TTV in cultured cells have been reported (5, 18, 24), it is important to construct an infectious TTSuV DNA clone so that TTSuV can be used as a useful model to study the replication and transcription mechanisms and to dissect the structural and functional relationships of anellovirus genes. More importantly, the availability of a TTSuV infectious DNA clone will afford us an opportunity to use the pig as a model system to study the replication and pathogenesis of TTSuV or even human TTV.

In the present study, we describe the construction and initial characterization of full-length DNA clones of TTSuV2 *in vitro* and *in vivo*. We provide, for the first time, definite evidence of splicing of TTSuV2 mRNA and expression of the putative ORF1 capsid protein by transfection of the TTSuV2 full-length DNA clones in cultured cells. Furthermore, rescue of TTSuV2 containing the introduced genetic markers in pigs was confirmed by sequencing of viral DNA obtained from pigs experimentally inoculated with the circular TTSuV2 genomic DNA.

## MATERIALS AND METHODS

**Cell lines and cell cultures.** A total of 12 continuous cell lines were used in this study. PCV1-free porcine kidney epithelial cell line PK-15 (8), swine testis cell line ST (ATCC CRL-1746, passage 6), baby hamster kidney fibroblast cell line BHK-21 (ATCC CCL-10, passage 62), and an African green monkey kidney epithelial Vero cell line (ATCC CCL-81, passage 95) were each grown in modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Porcine monocytic cell line 3D4/31 (ATCC CRL-2844, passage 8), porcine small intestinal epithelial cell line IPEC-J2 (a gift from Anthony Blikslager at North Carolina State University, Raleigh, NC) (40), and hamster ovary cell line CHO-K1 (ATCC CCL-61, passage 12) were each cultured in Dulbecco's modified Eagle's medium (DMEM) and nutrient mixture F-12 (Ham) (1:1) with GlutaMAX-I (Invitrogen, Carlsbad, CA) supplemented with 5% FBS and antibiotics. Monkey kidney cell line subclone MARC-145 (passage 42) derived from MA-104 (ATCC CRL-2378), human cervical cancer cell line HeLa (ATCC CCL-2, passage 10), and human hepatocellular carcinoma cell lines Huh-7 (subclone 10-3; a gift from Suzanne U. Emerson at NIAID, NIH) (7) and HepG2 (ATCC CRL-10741, passage 7) were each grown in DMEM supplemented with 10% FBS and antibiotics. Human 293 cell line 293TT, engineered to stably express high levels of simian virus 40 (SV40) large T antigen (a gift from John T. Schiller, Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, MD) (4), was cultured in DMEM-10 medium (DMEM with 10% inactivated FBS, 1% nonessential amino acids, and 1% GlutaMAX-I) supplemented with 400 µg/ml hygromycin B and antibiotics. All cells were grown at 37°C with 5% CO<sub>2</sub>.

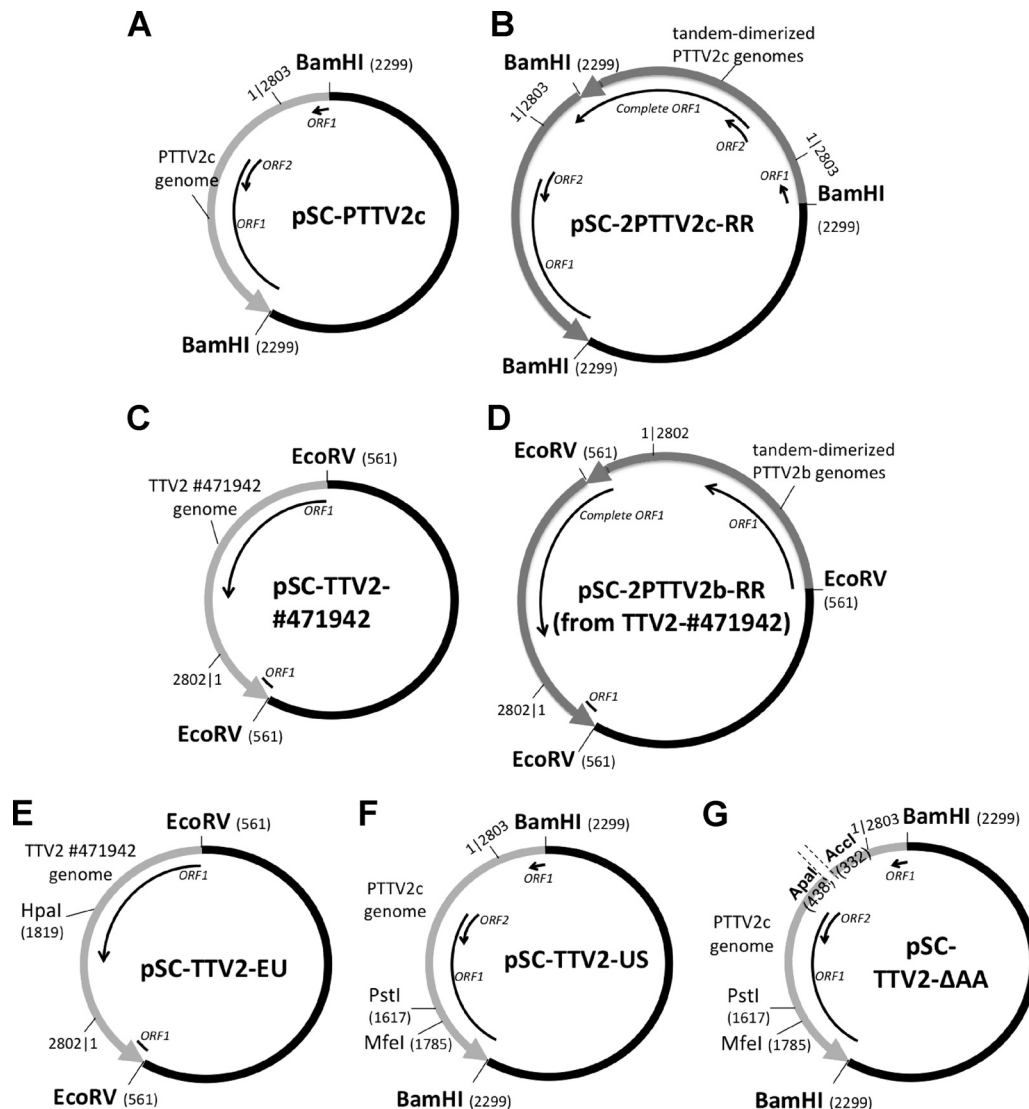
**Analysis of TTSuV1 or TTSuV2 contamination in cultured cells by real-time quantitative PCR (qPCR).** To ensure that the swine-derived cell lines used in this study were free of TTSuV contamination, five cell lines, PCV1-free PK-15, 3D4/31, IPEC/J2, BHK-21, and MARC-145, were tested for TTSuV1 or TTSuV2 DNA by using two singleplex SYBR green-based real-time qPCR assays (14). Briefly, total DNA was extracted from each cell line using the QIAamp DNA minikit (Qiagen) and subsequently subjected to TTSuV1 or TTSuV2 qPCR detection in a 25-µl PCR system using the SensiMix SYBR & Fluorescein kit (Quantace Ltd.) as described previously (14). A TTSuV1 or TTSuV2 standard template and a porcine

serum sample from a commercial company used in cell culture, which is supposed to be OIE (The World Organization for Animal Health) disease free, were included as controls. We also tested if the swine-derived trypsin and FBS products used for cell cultures were contaminated with TTSuVs. Two trypsin products from two different manufacturers and an FBS product were tested, including one trypsin product that was used for passaging of the cell lines. The trypsin was heat inactivated at 65°C for 2 h before DNA extraction. All samples were run in duplicate on the same plate.

**Generation of rabbit anti-TTSuV2 ORF1 antiserum.** We have previously expressed and purified a recombinant truncated ORF1 protein of TTSuV2 (PTTV2c-VA strain) (15). The purified protein products were used to immunize two New Zealand White rabbits as a custom antibody production service at Rockland Immunochemicals (Gilbertsville, PA). Serum samples from both rabbits were collected before immunization (pre-bleed) and at 45 days postimmunization. The specificity of the TTSuV2 ORF1 antiserum was demonstrated by Western blot analysis using the TTSuV2 ORF1 antigen and the bacterial control (cell lysis product from bacteria harboring the empty expression vector), as well as experiments in which PK-15 cells were transfected (see below) with the TTSuV2 ORF1 expression construct and two empty vectors, pTriEx1.1-Neo and pSC-B-amp/kan (data not shown).

**Construction of full-length genomic DNA clones of TTSuV2.** Two PCR fragments (E and F) covering the full-length genome of the U.S. strain of TTSuV2 isolate PTTV2c-VA (GenBank accession no. GU456386) were reamplified from the constructs reported previously (16), which were subsequently assembled into a full-length genomic DNA by overlapping PCR using the Hercules II Fusion DNA Polymerase (Stratagene) in the vector pSC-B-amp/kan (Stratagene). The monomeric TTSuV2 DNA fragment was flanked by a BamHI restriction site at both ends. The resulting construct was designated pSC-PTTV2c (Fig. 1A). The full-length PTTV2c genome was excised from clone pSC-PTTV2c by BamHI digestion, purified, and ligated head to tail to form concatemers. Two-copy concatemers were cloned into the BamHI-predigested pSC-B-amp/kan vector to produce a tandem-dimerized TTSuV2 DNA clone, pSC-2PTTV2c-RR (Fig. 1B). Similarly, two plasmids harboring monomeric and tandem-dimerized TTSuV2 genomic DNA from German TTSuV2 isolate TTV2-#471942 (GenBank accession no. GU188046) (9) were constructed with the EcoRV site on the same vector backbone, respectively. The monomeric TTSuV2 DNA fragment was flanked by an EcoRV restriction site at both ends (Fig. 1C and D). Since the TTV2-#471942 strain was classified into TTSuV2 subtype 2b together with U.S. isolate PTTV2b-VA based upon phylogenetic analysis (data not shown), we designated these two clones pSC-TTV2-#471942 (Fig. 1C) and pSC-2PTTV2b-RR (Fig. 1D), respectively.

**Introduction of genetic markers into the two TTSuV2 monomeric DNA clones and construction of a TTSuV2 deletion mutant.** An HpaI restriction enzyme site was engineered into the putative spliced region (intron) of the TTSuV2 genome in clone pSC-TTV2-#471942 for the introduction of a genetic marker to discriminate between the cloned virus and the potential indigenous viruses in the subsequent animal study. To create the unique HpaI site (GTTAAG; mutations are underlined), three point mutations, C to T, C to A, and T to A at nucleotide positions 1817, 1819, and 1820, corresponding to the TTV2-#471942 genome, were generated by a fusion PCR technique using two pairs of primers containing the desired mutations. The fusion PCR product was used to replace the corresponding region in clone pSC-TTV2-#471942 by using the KpnI cloning sites at both ends. The mutations were located in the putative intron of the TTV2-#471942 genome and did not change the putative ORF1 capsid amino acid sequence. The resulting full-length DNA clone was named pSC-TTV2-EU (Fig. 1E). Using the same strategy, two unique restriction sites, PstI (CTGCAG) and MfeI (CAATTG), were introduced into the putative intron of the PTTV2c-VA genome in the pSC-PTTV2c clone (Fig. 1F). The new clone, designed pSC-TTV2-US, contained three silent mutations at nucleotide positions 1613 (A to T), 1784 (T to C), and 1787 (C to T), corresponding to the PTTV2c-VA genome. A mutant clone pSC-TTV2-ΔAA, with a 104-bp deletion (nucleotide positions 332 to



**FIG 1** Schematic diagrams of TTSuV2 constructs containing full-length TTSuV2 genomic DNA. (A) pSC-PTTV2c (from U.S. TTSuV2 isolate PTTV2c-VA [GenBank accession no. [GU456386](#)]). (B) pSC-2PTTV2c-RR (tandem-dimerized PTTV2c-VA genomes). (C) pSC-TTV2-#471942 (from German TTSuV2 isolate TTV2-#471942 [GenBank accession no. [GU188046](#)]). (D) pSC-2PTTV2b-RR (tandem-dimerized TTV2-#471942 genomes). (E) pSC-TTV2-EU (derived from pSC-TTV2-#471942). An HpaI site was introduced into this clone as a silent genetic marker. (F) pSC-TTV2-US (derived from pSC-PTTV2c). PstI and MfeI sites were introduced into this clone as silent genetic markers. (G) pSC-TTV2-ΔAA. A 104-bp deletion mutation was introduced between the AccI and ApaI sites ranging from the putative TATA box to the ORF1 start codon in clone pSC-TTV2-US. The restriction enzyme (BamHI or EcoRV) used for plasmid construction is shown. The nucleotide positions of the restriction enzymes corresponding to the genomic sequences, the termini of the virus genome, ORF1, and ORF2 are labeled. The plasmid backbone used for cloning was the pSC-B-amp/kan vector (indicated by black). Gray arrows indicate the TTSuV2 genomic copies.

437) from the putative TATA box to the ORF1/ORF2 start codon on clone pSC-TTV2-US, was also generated by removing the short deletion fragment by double digestion with the AccI and ApaI enzymes, followed by the formation of two blunt ends with the Klenow enzyme and self-ligation (Fig. 1G). All mutagenesis was confirmed by DNA sequencing.

**In vitro transfection of TTSuV DNA clones.** PCV1-free PK-15 cells were seeded at  $2 \times 10^5$  per well of a six-well plate and grown until 60 to 70% confluence before transfection. Two-microgram samples of tandem-dimerized clones pSC-2PTTV2b-RR and pSC-2PTTV2c-RR and vectors pTriEx1.1-Neo and pSC-B-amp/kan were directly transfected into the cells, respectively, using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. For monomeric clones pSC-PTTV2c, pSC-TTV2-#471942, pSC-TTV2-EU, pSC-TTV2-US, and pSC-TTV2-ΔAA, the respective genomic fragment was excised with the BamHI or EcoRV

enzyme, gel purified, and religated with T4 DNA ligase overnight. The ligation mixtures ( $\sim 2 \mu\text{g}$ ) were used for transfection using Lipofectamine LTX, respectively. Cells were cultured for 3 to 5 days and then subjected to an immunofluorescence assay (IFA) to detect the expression of ORF1. Alternatively, transfected cells were passaged into new six-well plates and cultured for 3 days before detection of ORF1 expression by IFA. Transfection of the other 11 cell lines and IFA detection were done similarly.

**IFA.** Transfected or passaged cells on six-well plates were washed two times with phosphate-buffered saline (PBS) and fixed with acetone. Five hundred microliters of anti-TTSuV2 ORF1 antiserum at a 1:500 dilution in PBS was added to the cells for each well and incubated for 1 h at room temperature. Cells were washed three times with PBS, and 500  $\mu\text{l}$  Texas Red- or Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) at a 1:300 dilution was subsequently added. After incubation for 1 h at room



temperature, the cells were washed with PBS, stained with 500  $\mu$ l 4',6-diamidino-2-phenylindole (DAPI; KPL, Inc.) at a 1:1,000 dilution, and visualized under a fluorescence microscope.

**Reverse transcription (RT)-PCR.** Total RNA was extracted from PCV1-free PK-15 cells transfected with circular TTSuV2 DNA or the cloning vector pSC-B-amp/kan using the RNeasy minikit (Qiagen), followed by RNase-free DNase I treatment. The cDNA synthesis was performed using SuperScript II reverse transcriptase (Invitrogen) with oligo(dT) as the reverse primer. PCR was performed in a 50- $\mu$ l reaction mixture with the Advantage 2 PCR kit (Clontech) using primers TTV2-448F (5'-GAAGAAAGATGGCTGACGGTAGCGTACT-3') and TTV2-2316R (5'-AGGTGCTTGAGGAGTCGTCGCTTG-3'). The PCR products were gel purified, cloned into the pCR2.1 vector (Invitrogen) by the TA cloning strategy, and sequenced.

**In vivo transfection of colostrum-deprived (CD) pigs with tandem-dimerized TTSuV2 clones.** It has been previously demonstrated that the infectivity of infectious DNA clones of viruses with circular genomes can be tested by direct inoculation of dimerized full-length genomic DNA into animals (8). Therefore, in this study, a pilot animal study was initially conducted to determine the infectivity of tandem-dimerized TTSuV2 clones pSC-2TTV2c-RR and pSC-2TTV2b-RR. Briefly, six 26-day-old CD pigs that were seronegative and negative for TTSuV1 and TTSuV2 viral DNA were assigned to three groups of two each. Each group of pigs was housed separately and maintained under conditions that met all of the requirements of the Institutional Animal Care and Use Committee. The pigs in each group were injected by using a combination of intralymphoid (superficial inguinal lymph nodes) and intramuscular routes with the plasmid DNA of the full-length TTSuV2 clones. The two pigs (no. 1 and 2) in group 1 were each given 1 ml of PBS buffer and used as the negative control. The two pigs (no. 3 and 4) in group 2 were each injected with 200  $\mu$ g of pSC-2TTV2c-RR plasmid DNA, and the two pigs (no. 5 and 6) in group 3 were each inoculated with 200  $\mu$ g of the pSC-2TTV2b-RR clone.

Pigs were monitored daily for evidence of TTSuV2 infection for a total of 44 days. All pigs were necropsied at 44 days postinoculation (dpi). Serum samples were collected from all of the pigs prior to inoculation and weekly thereafter until termination of the study. The samples were tested for the presence of TTSuV DNA and viral loads were quantified by a singleplex TTSuV2-specific real-time qPCR (14). Brain, lung, lymph node, liver, kidney, thymus, spleen, small intestine, large intestine, heart, tonsil, and bone marrow tissue samples were collected during necropsies and processed for microscopic examination. The tissues were examined in a fashion blinded to the treatment status of the pigs and given a subjective score for severity of tissue lesions ranging from 0 (normal) to 3 (severe) (8, 11).

**In vivo transfection of cesarean-derived CD (CD/CD) pigs with the circularized TTSuV2 genomic DNA containing genetic markers.** To further verify the results of the initial pilot pig study, we introduced tractable genetic markers into the full-length DNA clones and conducted another CD/CD pig study. Approximately 600  $\mu$ g of circular or concatemeric TTSuV2 genomic DNA derived from clone pSC-TTV2-EU or pSC-TTV2-US was generated by ligation of the linearized TTSuV2 genomic DNA. To determine the infectivity of the full-length DNA clones, we inoculated each of four 40-day-old CD/CD pigs (no. 129, 135, 139, and 140 in group 1) with 150  $\mu$ g of concatemeric TTV2-EU DNA by a combination of the intralymphoid and intramuscular routes. Another four CD/CD pigs (no. 133, 137, 138, and 141) in group 2, which were housed in a separate room, were each similarly inoculated with 150  $\mu$ g of concatemeric TTV2-US DNA. The remaining four CD/CD pigs (no. 127, 132, 136, and 142) in group 3 were each injected with 1.5 ml of PBS buffer and served as negative controls. All pigs were monitored for evidence of TTSuV2 infection for 35 days and then necropsied. Total DNA was extracted without DNase I pretreatment from all samples and with DNase I pretreatment from selected samples. Viremia was tested by a TTSuV2 real-time qPCR (14). A TTSuV2 genomic region of 620 bp containing the engineered genetic markers in TTV2-EU or TTV2-US was

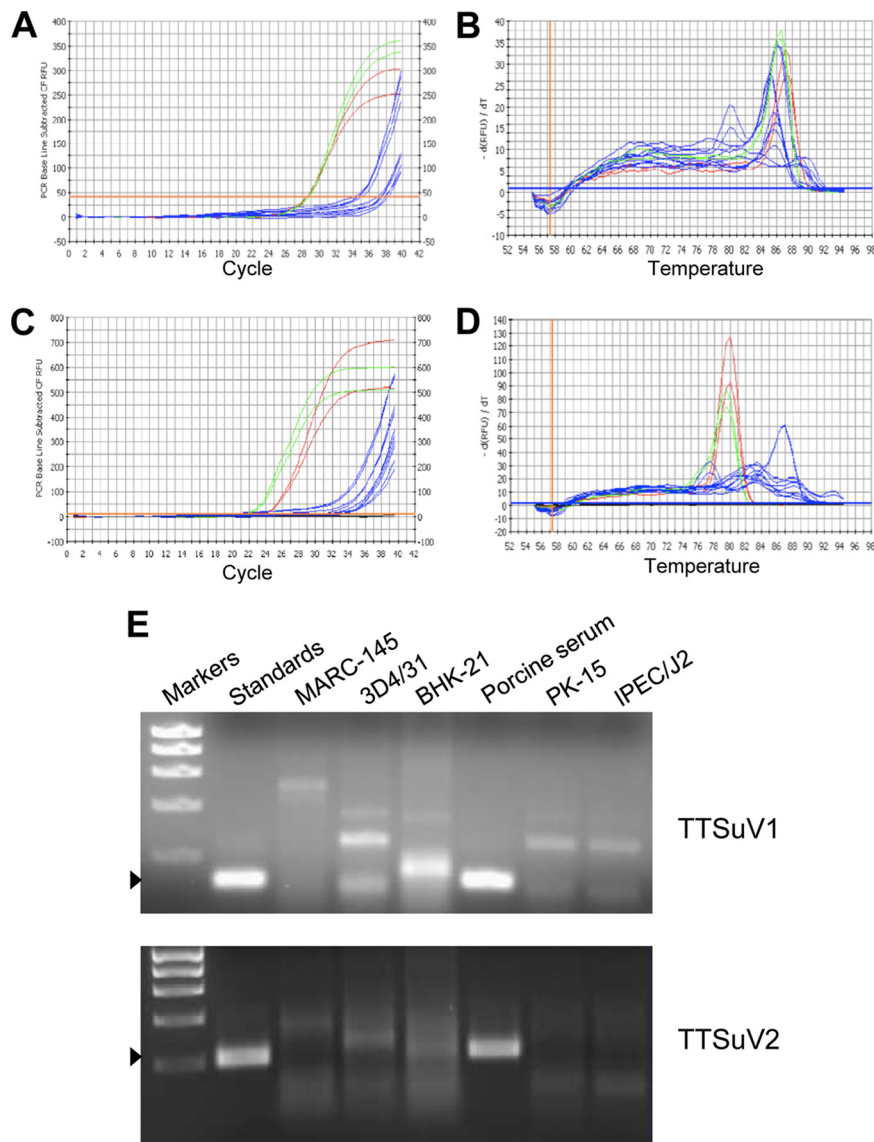
amplified from the sera of inoculated pigs by PCR using primers TTV2-tagF (5'-TGACACAGGA/CGTAGGAAATGCAGT-3') and TTV2-tagR (5'-TGAAGTATTTAGGGTCATTTGTAGCA-3') from selected serum samples of pigs with viremia. The PCR products were gel purified and cloned into a pCR2.1 vector by the TA cloning strategy. The white bacterial clones on the 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal)-containing agar plates were picked up for subsequent DNA extraction and sequencing.

## RESULTS

**Neither the viral DNA nor the expression of the putative ORF1 capsid protein of TTSuV1 or TTSuV2 was endogenously present in the five representative cell lines tested in this study.** The present study first aimed to identify potential permissive cell lines supporting TTSuV propagation. We selected five commonly used cell lines, including three that are of pig origin: PCV1-free PK-15, 3D4/31, and IPEC-J2 and two other cell lines, BHK-21 and MARC-145. These cell lines are known to be permissive to a wide variety of animal virus infections. In order to rule out the possibility of endogenous contamination of cultured cell lines with TTSuV1 or TTSuV2, the expression of both viral DNA and ORF1 protein was subjected to TTSuV1 or TTSuV2 real-time qPCR and IFA detection, respectively. OIE disease-free porcine serum that had been shown to have a high level of anti-TTSuV2 ORF1 antibody was also included as a control (15). The results of the qPCR analysis showed that none of the five cell lines tested in this study were positive for TTSuV1 or TTSuV2 DNA, as determined by the analyses of fluorescence curves, melting curves, and agarose gel electrophoresis, since their fluorescence curves were below the minimum detection limit, their melting curves did not overlap those of the standards, and there were no specific bands corresponding to the expected PCR products (Fig. 2). The bands from the cell samples close in size to the standards were excised from the gel, sequenced, and found to be porcine and mammalian genomic sequences. The swine-derived trypsin and FBS products used for cell cultures were also negative (data not shown). In contrast, as expected, the commercial porcine serum was positive for TTSuV1 and TTSuV2 DNA (Fig. 2).

To develop cell-based serological methods such as IFA or immunoperoxidase monolayer assay for TTSuV detection, we raised three specific antisera against the putative ORF1 capsid protein of TTSuV1a, TTSuV1b (Huang et al., submitted), or TTSuV2 in rabbits. When the five cell lines were stained with each of the three virus-specific antisera, respectively, no positive fluorescence signals were detected, indicating the absence of endogenous TTSuV1 or TTSuV2 ORF1 expression (data not shown). The IFA results were consistent with the qPCR detection, which demonstrated that the five selected cell lines were not contaminated with TTSuV1 or TTSuV2 and thus can be used to test for susceptibility to TTSuV infection or replication by transfection with TTSuV2 DNA clones.

**Construction and characterization of full-length TTSuV2 genomic DNA clones in porcine kidney PK-15 cells.** We were particularly interested in characterizing the infectivity of the TTSuV2 full-length DNA clone, since TTSuV2 has been reported to be associated with PMWS or PCVAD at a high rate of viral DNA prevalence (20), a large viral load (1), and a low antibody level in disease-affected pigs with an unknown mechanism (15). We first generated two monomeric full-length TTSuV2 DNA clones, pSC-PTTV2c and pSC-TTV2-#471942, derived from prototype U.S. isolate PTTV2c-VA and German isolate TTV2-#471942, respec-

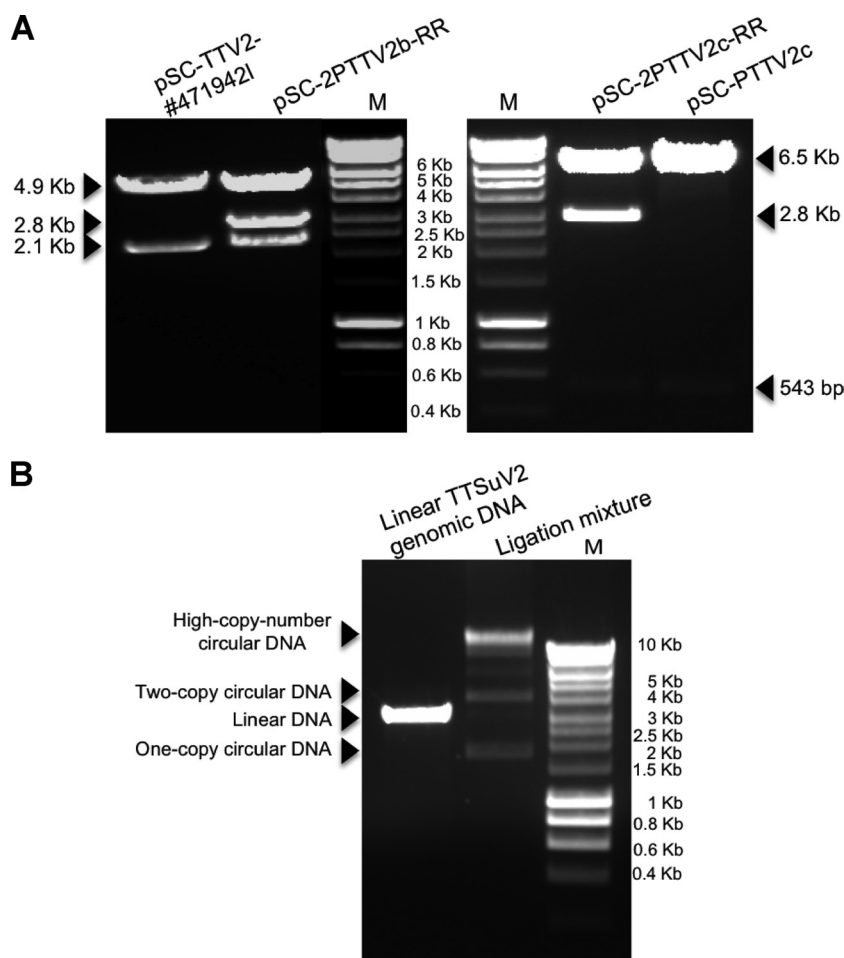


**FIG 2** Detection of TTsuV1 or TTsuV2 contamination in five different cell lines (PCV1-free PK-15, 3D4/31, IPEC/J2, BHK-21, and MARC-145) and an OIE disease-free porcine serum by real-time qPCR. Fluorescence curves (A and C) and melting curves (B and D) of TTsuV1 (A and B) or TTsuV2 (C and D) qPCR products are shown after 40 cycles of amplification of the standard template with the minimum dilution limit ( $10^{-4}$  pg; indicated by red), five different cell lines (blue) and the porcine serum (green). For each sample, duplicate determinations were made. (E) Detection of specific TTsuV1 or TTsuV2 qPCR products (marked by black arrowheads) by agarose gel electrophoresis.

tively (Fig. 1A and C) (9, 16). Each full-length TTsuV2 genomic DNA was inserted into cloning vector pSC-B-amp/kan, which does not contain a eukaryotic promoter. BamHI or EcoRV is the unique restriction site in the PTTV2c-VA or TTV2-#471942 genome, which was engineered at both ends of the genomic DNA to facilitate the generation of concatemers and thus to mimic the TTsuV circular DNA genome. Single digestion of the plasmid DNA of each clone with BamHI or EcoRV resulted in two different fragments of 4.3 kb and 2.8 kb. The 4.3-kb fragment represented the backbone vector, whereas the 2.8-kb fragment represented the inserted monomeric TTsuV2 genomic DNA (data not shown).

Subsequently, two copies of the full-length PTTV2c-VA genome from clone pSC-PTTV2c were ligated in tandem into the pSC-B-amp/kan vector to generate clone pSC-2PTTV2c-RR (Fig.

1B). Comparison of the AflII single-digestion patterns of pSC-PTTV2c and pSC-2PTTV2c-RR showed that the latter clone had an additional 2.8-kb fragment representing the intact single TTsuV2 genomic DNA (Fig. 3A, right side). We utilized the same cloning strategy to produce a tandem-dimerized TTsuV2 DNA clone, pSC-2PTTV2b-RR, derived from pSC-TTV2-#471942 (Fig. 1D). The construct pSC-TTV2-#471942 has two HindIII sites; one is located in the TTsuV2 genome, and the other is located in the vector. The short distance between them is 2.1 kb. When digested with HindIII alone, the construct pSC-TTV2-#471942 produced two fragments of 2.1 and 4.9 kb, whereas the construct pSC-2PTTV2b-RR gave an additional 2.8-kb fragment representing the intact single TTsuV2 genome (Fig. 3A, left side), thus confirming the successful construction of the clone.

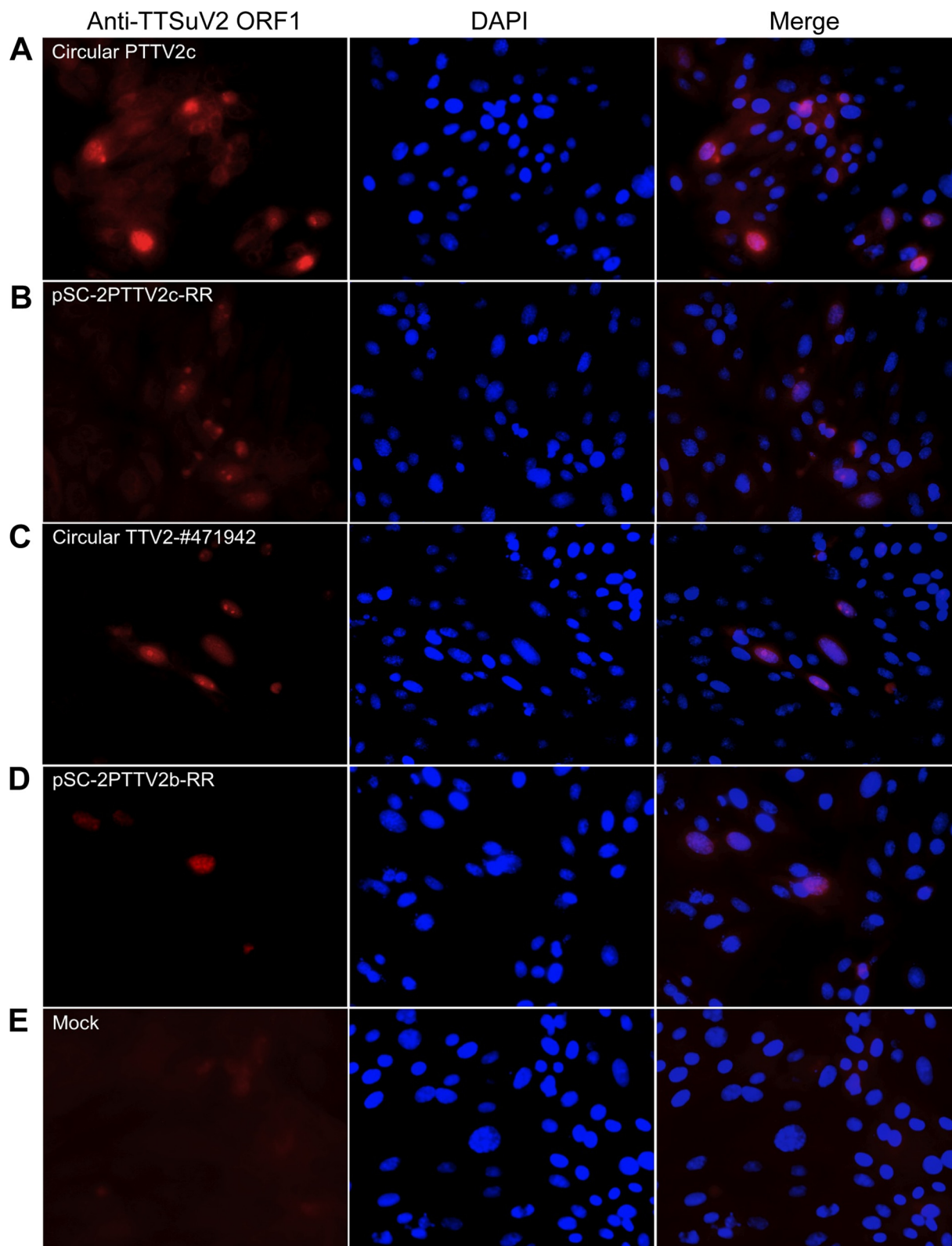


**FIG 3** Identification and quality assessment of linear or circular TTSuV2 genomic DNA. (A) Comparisons of the HindIII single-digestion patterns between clones pSC-TTV2-#471942 and pSC-2PTTV2b-RR (left side) and AflIII single-digestion patterns between clones pSC-PTTV2c and pSC-2PTTV2c-RR (right side) by agarose gel electrophoresis. Lanes M, DNA markers. The results were consistent with the predicted patterns of the digested fragments (shown by black arrowheads). The 2.8-kb fragments indicate the intact single TTSuV2 genomic DNA from clone pSC-2PTTV2b-RR or pSC-2PTTV2c-RR. (B) Quality assessment of concatemerized ligation products of the BamHI-digested and purified PTTV2c genomic DNA. Samples were electrophoresed in a 1% agarose gel before (linear DNA) and after (ligation mixture) T4 DNA ligase treatment. Linear DNA (~2.8 kb) and the putative one-copy (monomer), two-copy (dimer), and high-copy-number circular DNAs formed are indicated by arrowheads.

Circular TTSuV2 DNA was generated by tandem ligation of the purified linear TTSuV2 genomic DNA excised from clone pSC-PTTV2c or pSC-TTV2-#471942. Typical monomer, dimer, and high-copy-number molecules of concatemerized TTSuV2 DNA were observed in the ligation products (Fig. 3B). The ligation mixture from PTTV2c-VA or TTV2-#471942 was transfected into PCV1-free PK-15 cells. IFA conducted at 5 days posttransfection using rabbit antiserum against PTTV2c-VA ORF1 indicated that the TTSuV2 ORF1 antigen was expressed in the nuclei of the transfected cells with an approximately 5% positivity rate (Fig. 4A and C). No fluorescent signal was observed in mock-transfected cells stained with the same anti-TTSuV2 serum (Fig. 4E) or in circular TTSuV2 DNA-transfected cells stained with the anti-TTSuV1a ORF1, anti-TTSuV1b ORF1 (Huang et al., submitted), or prebleed rabbit serum (data not shown). Passage of the transfected cells two times did not eliminate but did reduce the fluorescent signal (data not shown). When the transfected cells were continuously passed for up to 20 passages, no positive signal was detectable, suggesting that TTSuV2 infection did not occur (data not shown).

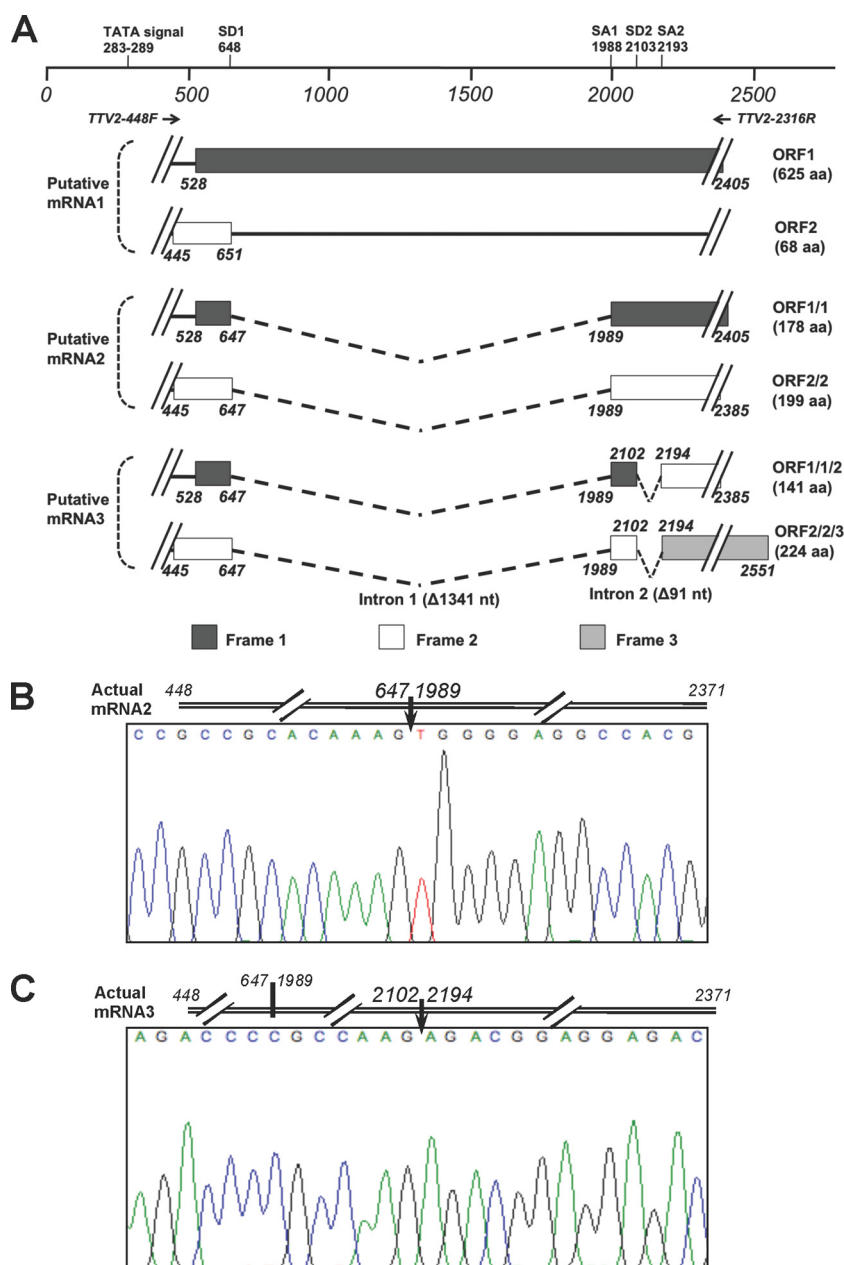
We next tested whether direct transfection of plasmid DNA of tandem-dimerized clone pSC-2PTTV2c-RR or pSC-2PTTV2b-RR into PK-15 cells resulted in the synthesis of TTSuV2 ORF1. A transfection efficiency of 50 to 60% in PK-15 cells was measured by using a green fluorescent protein (GFP) expression construct with the Lipofectamine LTX reagent (data not shown). The tandem-dimerized double-stranded DNA does not represent genomic anellovirus DNA but might represent an infectious replicative intermediate. IFA at 5 days posttransfection using the same anti-TTSuV2 ORF1 antiserum confirmed that both DNA clones also expressed ORF1 in transfected PK-15 cells (Fig. 4B and D). Again, ORF1 was expressed in cell nuclei. However, the fluorescence intensity and positivity rate were lower than those in circular TTSuV2 DNA-transfected cells (Fig. 4B and D). We did not observe localization of the ORF1 antigen in the cytoplasm of the transfected cells.

**Experimental identification of two introns in the TTSuV2 genome.** Although the transcriptional profile of cloned TTSuV full-length genomic DNA has not been reported, we previously speculated that TTSuV likely expresses two essential viral mRNA transcripts, mRNA1 and mRNA2, to produce the four known



**FIG 4** IFA results of PCV1-free PK-15 cells transfected with the ligation mixtures of linear TTSuV2 genomic DNA derived from clone pSC-PTTV2c (A) or pSC-TTV2-#471942 (C) with plasmid pSC-2PTTV2c-RR (B) or pSC-2PTTV2b-RR (D) or with Lipofectamine LTX only (E). Cells were stained with a rabbit anti-TTSuV2 ORF1 polyclonal antibody and Texas Red-conjugated goat anti-rabbit IgG (red) at 5 days posttransfection (left column). DAPI (blue) was used to stain the cell nuclei (middle column). The antibody and DAPI stainings are shown merged in the right column. Magnification,  $\times 200$ .

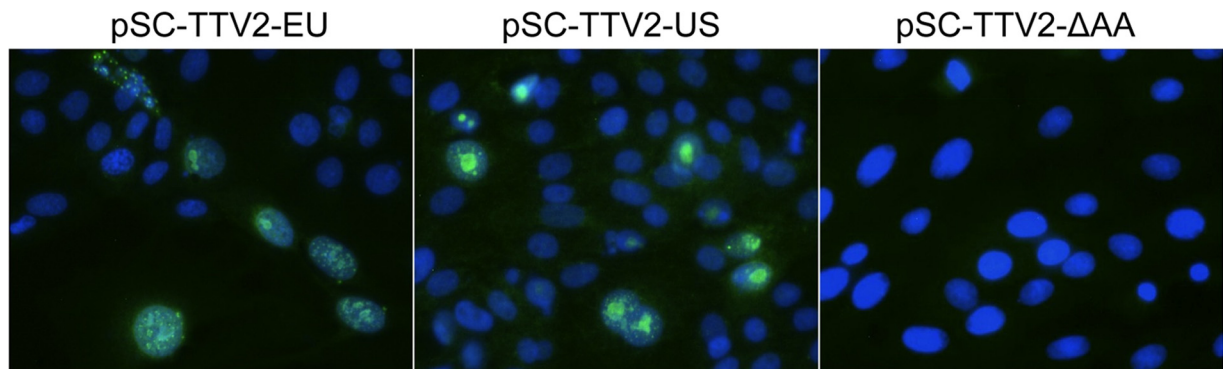




**FIG 5** Putative transcription profile and protein expression of TTSuV2 based on the PTTV2c-VA genome. (A) Schematic diagram of three putative viral mRNAs and six viral proteins. The TATA box, splicing sites (SD, splicing donor; SA, splicing acceptor), and positions of primers TTV2-448F and TTV2-2316R are indicated at the top. The three ORFs are depicted as boxes. The sizes of the six ORFs and two introns are also shown. aa, amino acids. (B) Sequencing of the RT-PCR products amplified by primers TTV2-448F and TTV2-2316R verified the splicing of putative intron 1. The actual viral mRNA2 is illustrated, and the nucleotide positions of both ends of mRNA2 and the splicing sites are marked. (C) Sequencing of the RT-PCR products amplified by primers TTV2-448F and TTV2-2316R identified an additional intron (intron 2). The actual viral mRNA3 is illustrated, and the nucleotide positions of both ends of mRNA3 and the splicing sites are marked. Arrows and numbers indicate the joint sites of the exons.

ORF counterparts of human TTV (Fig. 5A) (16). Continuous mRNA1 encodes ORF1 and ORF2, whereas removal of the putative intron of 1,341 nucleotides (nt) (designated intron 1 here), corresponding to nucleotide positions 648 to 1988 in the PTTV2c-VA genome, generates putative mRNA2, which contains two discontinuous ORFs, ORF1/1 and ORF2/2 (16). We also speculated that more spliced mRNAs and their encoded proteins of TTSuV may exist, as shown for human TTV (30, 38).

To determine whether the splicing of putative intron 1 in TTSuV2 occurred, total RNA was extracted from PK-15 cells transfected with circular PTTV2c-VA DNA, followed by DNase I treatment and RT-PCR analysis. Two PCR product bands of approximately 500 bp and 600 bp were visualized by agarose gel electrophoresis. Sequencing of the cloned PCR fragments resulted in the identification of two sequences. As expected, the large cDNA fragment of 583 bp was exactly the intron 1-spliced product



**FIG 6** IFA results of PCV1-free PK-15 cells transfected with the ligation mixtures of linear TTSuV2 genomic DNA derived from clone pSC-TTV2-EU, pSC-TTV2-US, or pSC-TTV2-ΔAA. Cells were stained with an anti-TTSuV2 ORF1 antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG (green) at 3 days posttransfection. DAPI (blue) was used to stain the cell nuclei. Only merged images of antibody and DAPI stainings are shown. Magnification,  $\times 200$ .

(Fig. 5B), whereas the small cDNA product of 492 bp contained two splicing regions, including intron 1 and an additional 91-nt intron, corresponding to nucleotide positions 2103 to 2193 in the PTTV2c-VA genome, which was designated intron 2 in this study (Fig. 5C). The splicing sites are conserved among all of the published TTSuV2 sequences (data not shown). Therefore, in this study, for the first time, we experimentally demonstrated the existence of splicing of intron 1 and the viral mRNA2 transcripts. We also identified a novel viral mRNA transcript, termed mRNA3, which encodes two putative proteins, ORF1/1/2 and ORF2/2/3, and which switches reading frames from 1 to 2 and 2 to 3, respectively, due to the splicing of intron 2 (Fig. 5A). The mRNA3 transcript contains at least three exons in the TTSuV2 genome. Since we failed to determine the 5' and 3' ends of the viral mRNA transcripts by rapid amplification of cDNA ends-PCR, it is possible that there exists an additional TTSuV2 intron in the region upstream of ORF2, as known in human TTV transcripts (30). However, the human TTV genome does not contain a short intron corresponding to TTSuV2 intron 2 in the region downstream of the large intron (intron 1).

Nevertheless, transfection of PK-15 cells with circularized TTSuV2 genomic DNA resulted in the synthesis of viral mRNA transcripts and expression of the ORF1 protein, indicating that the TTSuV2 concatemers likely mimicked the transcription and of protein expression from the natural circular genome of TTSuV2.

**A tandem-dimerized TTSuV2 clone, pSC-2PTTV2c-RR, is infectious when inoculated into CD pigs.** To test the infectivity of TTSuV2 DNA clones in pigs, we first performed a pilot study with three groups of CD pigs with two pigs per group. The pigs were inoculated with PBS buffer (pigs 1 and 2) in group 1, the tandem-dimerized clone pSC-2TTV2c-RR (pigs 3 and 4) in group 2, and pSC-2TTV2b-RR (pigs 5 and 6) in group 3, respectively. Serum samples were collected from the animals at 0, 7, 14, 21, 28, 35, and 42 dpi. Pig no. 2 died of septicemia due to an unidentified bacterial infection shortly after inoculation.

TTSuV2 DNA was detected by real-time qPCR beginning at 28 dpi in two pigs inoculated with pSC-2TTV2c-RR. The viral loads of both pigs, although very light, increased weekly until 42 dpi before necropsy at 44 dpi. The viral load in the serum of pig no. 3 increased from  $1.93 \times 10^3$  copies/ml at 28 dpi to  $5.59 \times 10^3$  copies/ml at 35 dpi and  $4.36 \times 10^4$  copies/ml at 42 dpi, whereas the serum viral loads in pig no. 4 rose from  $5.07 \times 10^3$  copies/ml at 28

dpi to  $4.49 \times 10^4$  copies/ml at 35 dpi and  $8.87 \times 10^4$  copies/ml at 42 dpi. Moderate microscopic lesions in the brain (lymphoplasmacytic encephalitis, mainly perivascular), liver (lymphohistiocytic hepatitis), and kidney (lymphoplasmacytic interstitial nephritis) were observed in pig 3 but not in pig 4. The remaining three pigs, including pigs inoculated with clone pSC-2TTV2b-RR, did not develop viremia during this study. However, pig no. 5 had mild lymphohistiocytic multifocal hepatitis. The results of this pilot pig experiment indicated that clone pSC-2PTTV2c-RR, from a U.S. strain of TTSuV2, is infectious.

**Characterization of two TTSuV2 full-length genomic DNA clones with engineered genetic markers and a derived mutant clone *in vitro*.** To further rule out possible contamination with other indigenous TTSuV2 strains in the pilot animal study, it is critical to introduce tractable genetic markers into the TTSuV2 genome so that the cloned virus and the potential indigenous contaminating virus in pigs can be discriminated in inoculated animals. We introduced a unique HpaI restriction site and two unique restriction sites, PstI and MfeI, into two TTSuV2 monomeric DNA clones, pSC-TTV2-#471942 and pSC-PTTV2c, to produce two new clones, pSC-TTV2-EU and pSC-TTV2-US, respectively (Fig. 1E and F). The positions of these sites, located in intron 1, were expected not to change the putative ORF1 capsid amino acid sequence. PK-15 cells were transfected with ligation mixtures of the linear TTSuV2 genomic DNA excised from these two marker clones, respectively. ORF1 expression in the nuclei of transfected cells was detected by IFA at 3 days posttransfection, similar to the patterns of their parental clones (Fig. 6), indicating that the clones with introduced genetic markers were replication competent.

Mutant clone pSC-TTV2-ΔAA, with a 104-bp deletion (nucleotide positions 332 to 437) from the putative TATA box (nucleotide positions 283 to 289; Fig. 5A) to the ORF1 (nt 528) and ORF2 (nt 445) start codons, was generated based on clone pSC-TTV2-US (Fig. 1G). When transfected into the PK-15 cells, the circularized DNA from this mutant clone did not express the ORF1 antigen (Fig. 6), suggesting that the deleted region likely contains a *cis*-acting element important for viral mRNA transcription or TTSuV2 ORF1 translation. The result obtained with the deletion mutant clone also implied that the observed expression of ORF1 is likely driven by replication-competent TTSuV2 DNA since the tandem-dimerized clone and concatemerized ligation

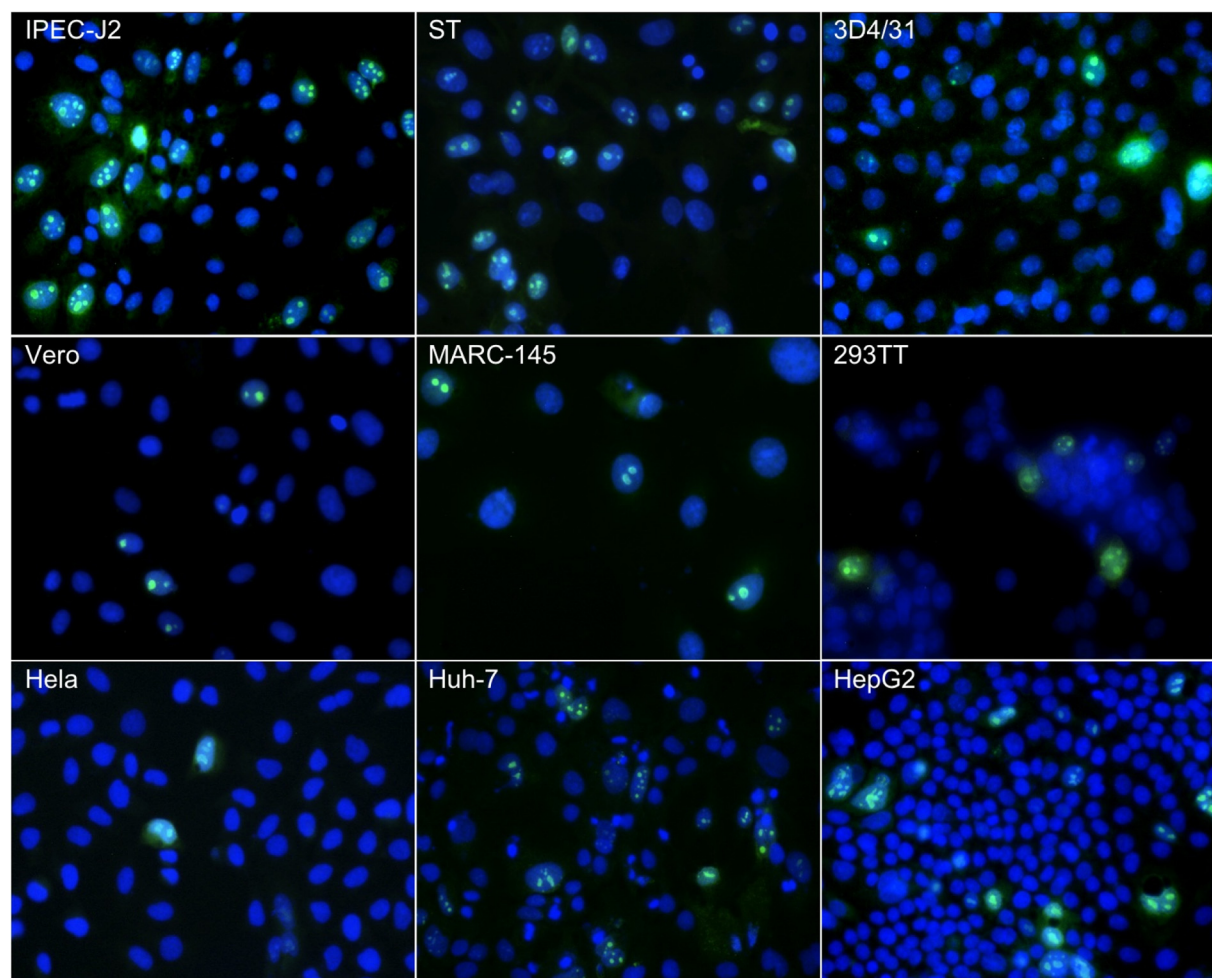


FIG 7 Transfection of nine different cell lines with the ligation mixture of linear TTsuV2 genomic DNA derived from clone pSC-TTV2-US. Images of Alexa Fluor 488-conjugated antibody staining (green) merged with nuclear staining with DAPI (blue) are shown. Magnification,  $\times 200$ .

products from the parental PTTV2c-VA genome were both infectious in pigs (see below).

**Expression of the TTsuV2 ORF1 protein in various cell lines transfected with the circularized TTsuV2 DNA from clone pSC-TTV2-US.** From the *in vitro* transfection experiments described above, it appeared that although the TTsuV2 putative ORF1 capsid protein is expressed, the PK-15 cells do not support the cell-to-cell spread of TTsuV2 recovered from the introduced TTsuV2 DNA clones. Alternatively, it is possible that the assembly of TTsuV2 virions in the transfected PK-15 cells is deficient. To search for another cell line that may be permissive for TTsuV2 infection, we subsequently transfected 11 other different cell lines with the circularized TTsuV2 DNA from clone pSC-TTV2-US, respectively. These cell lines included the four cell lines (3D4/31, IPEC-J2, BHK-21, and MARC-145) that were negative for TTsuV1 or TTsuV2 at both the DNA and protein levels. Plain cells of the other seven lines (ST, Vero, 293TT, HeLa, Huh-7, HepG2, and CHO-K1) were also negative for TTsuV2 ORF1, as determined by IFA (data not shown).

After transfection, all of the 11 cell lines expressed the ORF1 protein at 3 days posttransfection (Fig. 7; BHK-21 and CHO-K1 results not shown). The percentages of transfected cells with positive

IFA signals were subjectively categorized into the following three levels: IPEC-J2, ST, PCV1-free PK-15, Huh-7, and HepG2 with a high positivity rate ( $>5\%$ ); 3D4/31, Vero, MARC-145, and 293TT with a medium positivity rate (2 to 5%); and HeLa, BHK-21, and CHO-K1 with a low positivity rate ( $<2\%$ ). In general, TTsuV2-specific antibody staining patterns of individual positive cells by IFA could be divided into three different types: (i) cells displaying dense nuclear staining; (ii) cells displaying large nuclear inclusion staining; and (iii) cells displaying punctate nuclear staining. The last two patterns indicated the localization of ORF1 antigen in cell nucleoli. No cytoplasmic staining was observed in transfected cells.

To test if some of these IFA-positive cells were susceptible to TTsuV2 infection, supernatants collected from lysates of PK-15, ST, and 293TT cells transfected with circularized TTsuV2 DNA were inoculated into all of the cell lines with a high rate of positivity and some with a medium rate of positivity, including the 293TT cell line, respectively. The inoculated cells were cultured for 3 to 5 days and examined by IFA. No fluorescent signal was detected in these cells (data not shown), indicating that none of the cell lines tested are susceptible to TTsuV2 infection.

**Rescue of TTsuV2 from concatemeric TTsuV2 genomic DNA of clone pSC-TTV2-US in CD/CD pigs.** With the intro-



duced genetic markers in the full-length DNA clones that can be used to distinguish between infections caused by the cloned virus and potential indigenous contaminating virus, we performed an additional study with CD/CD pigs to further verify the *in vivo* infectivity of the TTSuV2 genomic DNA clones. Twelve CD/CD pigs were assigned to three groups of four pigs each. Pigs in each group were inoculated with PBS buffer, concatemericized TTV2-EU DNA, and TTV2-US DNA, respectively. Preinoculation serum samples from all of the pigs (collected 30 days prior to inoculation) were negative for TTSuV1 or TTSuV2 DNA by real-time qPCR. Serum samples were collected from all of the animals at 0, 7, 14, 21, 28, and 35 dpi.

TTSuV2 DNA was detected in all eight inoculated pigs, but unfortunately, it was also detected in two negative-control pigs (no. 136 and 142), indicating contamination with other strains of TTSuV2 indigenous in the research facility or the source pigs, which is not uncommon (see Table S1 in the supplemental material). One pig (no. 133) inoculated with the concatemericized TTSuV2-US DNA had detectable viremia even at 0 dpi, whereas the other pigs developed viremia at 14 or 21 dpi (see Table S1 in the supplemental material). Except for pig no. 133, the seven TTSuV2 DNA-inoculated pigs and the two TTSuV2-positive pigs in the negative-control group had increased viral loads until necropsy, indicating active virus infection (see Table S1 in the supplemental material). We speculated that the source of the TTSuV2 contamination was likely due to the 1-month waiting period between the date of preinoculation serum sample testing (by which all of the animals were negative) and 0 dpi. Since total DNA was extracted from these samples without DNase I pretreatment, to rule out the possibility of the presence of inoculated free DNA in the sera from pig no. 133, serum samples from this pig and from pigs no. 136 and 142 in the negative-control group with positive PCR results were subjected to DNase I treatment or left treated before DNA extraction followed by qPCR. The result was consistent with Table S1 in the supplemental material, showing that there was no significant viral load difference between DNase I-treated and untreated samples (see Table S2 in the supplemental material).

However, thanks to the introduced genetic markers in the TTSuV2 DNA clones used in this study, we were still able to determine if the TTSuV2 DNA clones were infectious in pigs, which was the main objective of our study. Since we have previously demonstrated that a single pig can be infected with multiple strains of TTSuV2 and TTSuV1 (9, 16), prior infection or concurrent infection with an indigenous TTSuV2 strain should not interfere with the infection of pigs with the TTSuV2 DNA clones we intended to test in this study. To determine if the genetic markers of TTSuV2-EU or TTSuV2-US were present in viruses recovered from the sera of infected pigs with the mixed TTSuV2 infection status, we amplified and sequenced a 620-bp region containing the engineered genetic markers from selected samples at 35 dpi from both inoculated and negative-control pigs. The results showed that only the serum samples from pigs experimentally inoculated with the concatemericized TTSuV2-US DNA had TTSuV2 sequences identical to the introduced genetic markers PstI and MfeI, whereas serum samples from the negative-control group and from pigs inoculated with concatemericized TTV2-EU DNA did not contain any introduced genetic markers (data not shown). Therefore, this pig study further confirmed the initial pilot pig study in which the TTSuV2-US full-length DNA clone was infectious in pigs. The

results also experimentally verified, for the first time, that pigs can be coinfecting with different strains of TTSuV2.

## DISCUSSION

Little is known about the biology and pathogenicity of anelloviruses due to the lack of a cell culture system to propagate human TTV or TTSuV and the lack of a suitable animal model combined with reverse genetic systems for anellovirus studies. Reports of TTSuV DNA sequences detected in commercial porcine vaccine products, in swine-derived human drugs, and in swine-derived trypsin by nested PCR suggested widespread contamination with TTSuV (19, 22). Cell cultures may be one of the major sources of TTSuV contamination in biological products of pig origin. Therefore, the present study was first aimed at examining whether five selected cell lines harbor endogenous DNA and protein antigen of TTSuV1 or TTSuV2 and to further identify TTSuV-negative cell lines that are potentially permissive of TTSuV propagation.

Surprisingly, none of the five cell lines tested in this study were found to be positive for TTSuV1 or TTSuV2 DNA or ORF1 antigen (Fig. 2). Furthermore, screening of seven additional commonly used cell lines also yielded negative results, as determined by IFA detection (data not shown), indicating that TTSuV contamination in cell cultures is probably not as common as we originally thought. Our result was distinct from that of a recent study by a Brazilian group that reported TTSuV DNA contamination in 15 of 25 cell lines (41). In that study, the five cell lines that were also used in our study, i.e., PK-15, ST, BHK-21, Vero, and MA-104 cells (from which the MARC-145 cell line is derived) were shown to have detectable TTSuV1 and/or TTSuV2 sequences by the use of a one-round duplex PCR assay (41). It is unclear why there is such a major discrepancy between the results of our study and those obtained by the Brazilian group. A reliable approach to proving the presence of a contaminating virus in cell cultures used in biological products is to determine the cells' susceptibility to virus infection, which has been exemplified by PCV1 (2, 12, 25, 42). Theoretically, the possibility of TTSuV contamination in cell cultures is very low, since anellovirus has been shown to be extremely difficult to propagate *in vitro*. The present study utilized the (i) more sensitive qPCR assay (compared to the one-round PCR used by Teixeira et al. [41]); (ii) the IFA; and (iii) transfection of circular TTSuV genomic DNA into the cells as the positive control (see below) to demonstrate the absence of TTSuV at both the DNA and amino acid levels in 12 representative cell lines, including four of pig origin (PK-15, ST, 3D4/31, and IPEC-J2). Therefore, based on the results of this study, we conclude that, contrary to what some may believe, there is very little, if any, endogenous TTSuV contamination in well-established continuous cell lineages. Instead, the detection of contaminating TTSuV DNA sequences in biological products reported by other groups may be due to the swine-derived trypsin (19, 41) or serum products used. The former was not detected (data not shown), but the latter was actually confirmed in the present study for the first time (Fig. 2).

Subsequently, we demonstrated that all of these TTSuV-free cell lines supported TTSuV2 ORF1 expression by transfection with circular TTSuV2 genomic DNA or tandem-dimerized TTSuV2 plasmids (Fig. 4, 6, and 7). The TTSuV2 ORF1 protein was expressed in cell nuclei, especially in nucleoli, which is consistent with the localization of human TTV ORF1 in Huh-7 cells transfected with the circular full-length TTV genomic DNA by



immunoblotting with the ORF1-specific antibody (30). Most recently, it was also reported that TTSuV1 or TTSuV2 ORF1-GFP fusion protein expressed from the recombinant construct accumulated in the nucleoli of PK-15 cells (27).

In addition, in this study TTSuV2-specific mRNA splicing events were detected in transfected PK-15 cells by RT-PCR, indicating the synthesis of viral mRNA transcripts in the transfected cells. While we experimentally demonstrated the existence of two viral mRNA transcripts (mRNA2 and mRNA3) (Fig. 5), putative mRNA1, encoding full-length ORF1 of TTSuV2, was not detected (data not shown), which may suggest a smaller quantity and lower integrity of mRNA1 than of mRNA2 and mRNA3. In accordance with the result described by Martinez-Guinó et al., splicing of the 91-nt intron 2 sequence into mRNA3 also occurred posttranscription of the TTSuV2 ORF1-GFP fusion gene based on a non-full-length viral clone (27).

The synthesis of viral mRNA transcripts and the subsequent expression of ORF1 or ORF1-related viral protein in transfected cells were driven by the endogenous TTSuV2 promoter. The processes were also regulated by the unidentified *cis*-acting elements, as we showed in this study that deletion of a 104-bp sequence downstream of the TATA box completely eliminated ORF1 expression (Fig. 6). To our knowledge, this is the first demonstration of porcine anellovirus mRNA and protein expression and mutagenesis analysis based on the viral DNA concatemers produced from circularized viral genomes or a tandem-dimerized full-length clone.

It appeared that both the PTTV2c-VA and TTV2-#471942 DNA concatemers were replication competent when transfected into cells since they mimicked the natural TTSuV2 circular genome. However, the rescue of PTTV2c-VA (TTV2-US) but not TTV2-#471942 (TTV2-EU) was demonstrated in only two *in vivo* animal experiments. The major sequence difference between these two TTSuV2 strains was in the GC-rich region. It has been proposed that the GC-rich region in anelloviruses forms unique stem-loop structures that may play a significant role in viral replication (29, 35). Further in-depth mutagenesis analysis, which was beyond the scope of the present study, is required to explain this discrepancy between the two clones.

We also showed that although the three cell lines tested in this study (PK-15, ST, and 293TT) supported a limited level of TTSuV2 replication, the infection of these cells with TTSuV2, if any, was nonproductive since the supernatants of the transfected cells did not induce a second-round infection. Most recently, the 293TT cell line was shown to be susceptible to human TTV propagation due to its expression of SV40 large T antigen at a high level (5). The authors proposed that the human TTV genome contains a conserved octanucleotide in the UTR forming a stem-loop as the putative origin of replication. Five 4-bp motifs (CGGG and GGGC) were found adjacent to the stem-loop that may act as the recognition sites for the SV40 large T antigen to facilitate TTV replication (5). However, when we performed a sequence alignment analysis of the corresponding sequences among human TTV, TTSuV, torque teno canis virus (dog anellovirus), and torque teno felis virus (cat anellovirus), neither the conserved octanucleotide nor the 4-bp motif was identified in the latter three anelloviruses (data not shown). Therefore, the SV40 large T protein expressed in 293TT cells likely does not have the proposed helper effect on TTSuV replication. Further study is needed to

determine whether additional cell lines are permissive to TTSuV2 infection.

Previous studies by our group and others have demonstrated that, even under strictly controlled experimental conditions in research facilities, TTSuV-negative pigs can easily acquire TTSuV infection due to the ubiquity of this virus in pigs and environments (10, 15). Although our second *in vivo* experiment in the present study unfortunately “validated” these previous reports, our results did demonstrate the successful rescue of TTSuV2 in pigs inoculated with either the tandem-dimerized plasmids or circular TTSuV2 DNA with the introduced genetic markers. Unfortunately, due to the presence of indigenous TTSuV2 in the CD/CD pigs from the second animal study, we could not analyze or correlate any pathological lesions in the inoculated pigs with TTSuV infection. Therefore, a future study using germfree gnotobiotic pigs and the infectious DNA clone is warranted to characterize the pathological lesions attributable solely to TTSuV2 infection. In addition, serum samples containing known loads of the rescued TTSuV2-US strain can be used as virus stocks for experimental infection of TTSuV2-negative pigs in future studies. The availability of the pig model combined with the anellovirus reverse genetic system described in this report will facilitate future studies of porcine and even human anellovirus biology and pathogenesis.

## ACKNOWLEDGMENT

This study was funded by a grant from Boehringer Ingelheim Vetmedica, Inc., Ames, IA.

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